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(57) Abstract <p>Cationic liposomes with entrapped polynucleotide in the intravesicular space are described. The liposomes include cationic components such as cationic lipids such as DOTAP. Preferably the method of forming liposomes uses the dehydration-rehydration method in the presence of the polynucleotide. The polynucleotide preferably operatively encodes an antigen capable of eliciting a desired immune response, that is, is a gene vaccine.</p>			

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LIPOSOMES

The present invention relates to compositions of liposomes with entrapped polynucleotide encoding a desired polypeptide. The polypeptide preferably encodes an immunogenic polypeptide useful to induce a desired immune response in a subject, for instance for prophylactic immunisation against infective microbes or for immunotherapeutic treatment. The liposomes preferably include at least one cationically charged lipid and are preferably made by a dehydration-rehydration technique.

It is known to introduce genetic material into the body of a human or animal subject for various purposes. In the mid 1960s it was suggested the technique could be used for the treatment of genetic diseases by introduction of a normal gene sequence into cells of a person carrying its defective counterpart. Trials are currently underway of methods of treating various inherited genetic disorders by gene therapy. For instance, a considerable amount of work has been carried out on the treatment of cystic fibrosis, by introducing DNA encoding the CF transmembrane conductance regulator. Since the gene product is required in the lungs, attempts have been made to deliver the gene directly into the lungs or intranasally.

More recently gene therapy has been proposed for treatment of cancer. For instance, by introducing genes encoding tumour necrosis factor (TNF) or interleukin-2 into lymphocytes or into tumour cells, it is hoped to stimulate an immune response resulting in tumour destruction. In addition, by introducing genes encoding a human class 1 major histocompatibility antigen (HLA-B7) into tumour cells of patients who do not express this antigen, it is hoped to stimulate an immune response to the antigen resulting in destruction of tumour cells.

A variety of vectors have been proposed for the delivery and expression of nucleic acids in gene therapy (Mulligan, 1993). They include viruses (eg. retroviruses and adenoviruses) as well as non-viral vectors (eg. cationic polymers and vesicles). However, there are

disadvantages with each of these vectors, for instance possible side effects upon integration of retroviruses into the cell genome, promotion of immune responses against viral proteins thus precluding long term treatment (Kay et al, 1994), and transient or low efficiency transfection by non-viral vectors (Legendre and Szoka, 1995). Nonetheless, the relative simplicity of DNA incorporation into non-viral vectors, often regardless of DNA size and structure, and their non-pathogenic nature, render these vectors an attractive alternative. Indeed, constructs (complexes of preformed cationic vesicles and plasmid DNA) have been now developed which exhibit high indexes of transfection in vitro (Felgner, 1991) and a low to modest transfection in experimental animals (Alton et al, 1993; Zhu et al, 1993). On the other hand, because of the potential toxicity (Raz et al, 1994) of such complexes and inability to incorporate other agents which may promote DNA transfer efficiency, their usefulness *in vivo* may not be as promising as when nucleic acids are incorporated within conventional liposomes. These, when appropriately designed (in terms of vesicle size, surface charge and lipid composition), remain stable in the blood circulation (Scherphof et al, 1983; Gregoriadis, 1995) thus protecting their nucleic acid contents from nucleases in blood plasma, or attain clearance rates conducive to optimal use (Gregoriadis, 1995). Moreover, grafting of cell-specific ligands to the surface of long circulating liposomes would direct nucleic acids preferentially to target cells (Gregoriadis, 1995). Incorporation of other agents into nucleic acid-containing liposomes may also render them fusogenic, facilitate escape of their contents from the endosomes into the cytoplasm or promote DNA transport into the nucleus (Legendre and Szoka, 1995). However, most techniques (Gregoriadis, 1993) for the entrapment of DNA into liposomes are inefficient, incompatible with its size or employ conditions (eg. sonication, organic detergents and solvents) which may be detrimental to DNA integrity.

WO-A-9117424 (Vical, Inc) describes various complexes of positively and negatively charged lipid species and an active compound with improved intracellular delivery. It is suggested that low rates of entrapment of polynucleotide into cationic liposomes can be overcome by a method in which a net positively charged complex of preformed positively charged liposomes and polynucleotides (which are negatively charged) is subsequently associated with an excess of preformed negatively charged liposomes which are said to coat the positively charged complex. However, since the polynucleotide is not entrapped inside any vesicle, it is believed that it will be accessible to nucleases in plasma.

In US-A-4,897,355 Eppstein et al (Syntex) describe liposomes formed of cationic lipid for use in intracellular delivery of active compounds. One example of active compound is a polynucleotide, for instance encoding enzymes for use in enzyme deficiency conditions, hormones for use in hormone replacement therapy, blood coagulation factors, neuro transmitters, anti-viral compounds and anti-cancer compounds or for delivery of anti-sense RNA for selectively turning off expression of certain proteins. The active compounds are admixed with preformed, empty liposomes and optionally the conjugate is subsequently admixed with further preformed liposomes.

In Journal of Drug Targeting, 1996, (in press) Gregoriadis et al described formation of liposomes with entrapped DNA for use in gene therapy made using a dehydration-rehydration technique.

At the 1995 conference "Targeting of Drugs: Strategies for Oligonucleotide and Gene Delivery in Therapy" and in the subsequent proceedings of that conference, eds. G. Gregoriadis and B McCormack (published 1996, Plenum Press, New York) Davis discusses undesirable immune response against products of genes introduced into cells for gene therapeutic processes. She suggests how the study of the immune response induced upon intracellular introduction of

genes is important to optimize gene therapeutic treatments as well as DNA-vaccination (DNA-based immunisation) applications of gene transfer. She describes many advantages of gene vaccines over antigen-based vaccines and 5 describes some results on the use of naked DNA for DNA based immunisation to Hepatitis B surface antigen (HBsAg).

At the same conference, Behr described synthetic carriers for polynucleotide sequences for gene therapy consisting of lipopolyamines which are alleged to self-10 assemble around DNA while condensing it. The polynucleotide is intended to reach the cell nucleus. The self-assembled particles are believed by the present inventor not to be constituted by a bilayer, and thus would not be defined as liposomes.

15 The pre-conference information relating to IPC's second annual conference "Genetic Vaccines and Immunotherapeutic Strategies", which took place on 23 and 24 October 1996 in Washington DC, USA, indicated Felgner would describe recent work using cationic lipid to improve 20 delivery of genes coding for antigens. The genes are delivered intranasally and into lung tissue to stimulate mucosal immunity. At the same conference, it was asserted that Ledley would describe gene delivery systems comprising cationic lipids to control the bioavailability and entry of 25 DNA into mucosal cells. The intention is to engineer an effective immune response. The pre-conference announcement gives no more information about how the gene delivery has been carried out.

It would be desirable to increase the encapsulation 30 rate of polynucleotides in liposomal delivery systems. Furthermore it would be desirable to increase the level of gene product in the circulation of animals to whom the genes had been administered, especially for therapeutic products. It is, furthermore, desirable to increase the 35 rate of delivery of the gene to target cells, where the gene product is an antigen. It would furthermore be desirable to provide improved delivery of polynucleotides

encoding immunogenic polypeptides which are useful to induce a desired immune response.

A first aspect of the present invention provides a new composition comprising liposomes and, entrapped within the 5 liposomes, a polynucleotide operatively coding for an immunogenic polypeptide which induces a desired immune response in a human or animal subject.

In this specification the term entrapped means that the polynucleotide is the intravesicular space. Thus the 10 liposomes have been formed in the presence of the polynucleotide. This is to be contrasted to the prior art complexes of preformed liposomes and polynucleotides.

In this aspect of the invention, the product of the gene should be an antigen against which an immune response 15 is desired. The peptide thus includes one or more antigenic determinants of infectious microorganisms, such as viruses, bacteria or fungi. The method is of particular utility in immunisation against bacteria, fungi and viruses, especially influenza, HIV, Hepatitis B and 20 Hepatitis C. The gene product may therefore be HBsAg or Hepatitis C core protein or an influenza antigen or an antigenic HIV peptide or fragment. The invention is also of value where the gene product is one or more herpes simplex virus proteins, a cancer virus product, such as 25 SV40, or a cancer antigen, tuberculosis antigens and even antigens of more complex microorganisms, such as parasites such as malaria.

In this invention the target tissue for liposome uptake may be muscle, skin, liver, spleen cancer cells to 30 be destroyed, mucosal cells, such as in the nose or lungs or intestine and especially cells of the lymph nodes. The target cells for gene vaccines are generally antigen presenting cells. The composition may be administered systemically, for instance by IV injection, or may be 35 administered directly to the target tissue, for instance intranasally, intramuscularly or intradermally, or even transdermally or orally. The present invention has allowed

for the first time, successful generation of an immune response where the liposomes are administered subcutaneously. It is a preferred aspect of this invention, therefore that the composition is administered 5 subcutaneously.

In the present specification the term "liposome" refers to vesicles surrounded by a bilayer formed of components usually including lipids optionally in combination with non-lipidic components.

10 It may be desirable to provide the external surface of the liposome with a targeting moiety, for instance an antibody, suitable for recognising target tissue. For instance where the composition is administered into the circulation, cell specific ligands attached to the external 15 surface of the liposome would direct the nucleic acids to the target cells (Gregoriadis, 1995).

20 The gene should be present in a form such that the desired product can be produced in the target cell, and thus preferably includes regulatory elements that 25 facilitate expression in the target cells. The polynucleotide thus includes a promoter, as well as regions to mediate ribosome binding, and optionally also other regions which might enhance gene expression.

30 The polynucleotide may be RNA, but is preferably DNA. It is generally in the form of a plasmid, preferably a 25 substantially non-replicating plasmid, since for this aspect of the invention, transient activity over a period of weeks or a few months is generally appropriate.

35 In this aspect of the invention, the liposome forming components used to form the liposomes may include neutral, zwitterionic, anionic and/or cationic lipid moieties. These may be used in relative amounts such as to confer an overall charge on the liposome or, less preferably, the liposomes may have no overall charge. It is found that using lipid components such that the liposome has an overall positive charge can provide improved results in this aspect of the invention, in terms of giving an

increase immune response when used to deliver an antigen-encoding gene. In addition to components which are properly termed lipids (including glycerides and cholesterol), the liposome forming components may include 5 non-lipidic components (i.e. which are not naturally occurring lipids) such as non-ionic or cationic surface active agents.

According to a particularly preferred embodiment of 10 the invention, the new composition comprises liposomes formed from liposome forming components including at least one cationically charged component in an amount such that the liposomes have an overall positive charge.

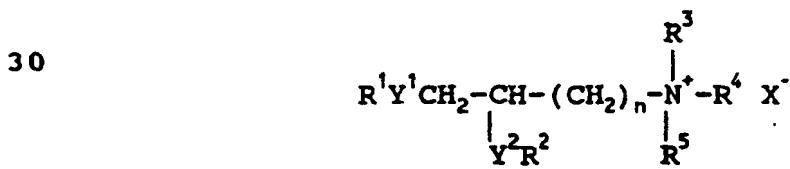
It is believed that this is the first time that 15 polynucleotide has been entrapped into a cationic liposome. Accordingly, in a second aspect of this invention there are 20 provided liposomes formed from liposome forming components including at least one cationically charged component and polynucleotide encoding a desired polypeptide product and is characterised in that the polynucleotide is entrapped within the liposome.

Thus the present inventor has established that in an 25 *in vitro* system, the entrapment of DNA encoding for luciferase marker protein gives increased levels of luciferase expression as compared to other methods using uncharged liposomes or anionically charged liposomes. Whilst the levels of expression were lower than using a complex of preformed cationically charged liposomes (the commercially available LipofectAMINE (trade mark)), it is 30 expected that an improvement in resistance to nuclease attack by the entrapment as compared to the complex would be exhibited *in vivo*. In addition it is found that the liposomes do not aggregate rapidly, whereas such aggregation can occur for mixed preformed liposomes - 35 polynucleotide systems especially in the presence of serum proteins. The entrapment of polynucleotide provides greater freedom to provide targeting ligands on the liposome surface or carrying out other surface treatments

on the liposomes with entrapped actives. It is expected that the high level of expression of the model protein luciferase would be exhibited where the gene product was an antigen inducing a desired immune response.

5 This has been confirmed by experiments which have shown that vaccination of mice with liposome-entrapped pRc/CMV HBS (encoding the S region of hepatitis B surface antigen; subtype ayw) by a variety of routes results in humoral and cell-mediated immune responses that are 10 independent on whether or not mice are inbred (Balb/c) or outbred (T/o) and route of injection (intramuscular, im; subcutaneous, sc; intravenous, iv; and intraperitoneal, ip). Such responses are in most cases significantly greater than those seen with naked DNA under identical 15 conditions (see example 5 below).

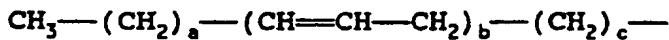
In this embodiment of the invention the cationic component incorporated into the liposome may be any of those which have been used in liposome preparations for improving transfection rate by complexation with 20 polynucleotides. The component may be a lipidic or a non lipidic compound and may be synthetic or natural. Preferred cationic lipids are, 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP), 1,2-bis(hexadecyloxy)-3-trimethylaminopropane (BisHOP), N-[1-(2,3-dioleyloxy)propyl]-N,N,N-triethylammoniumchloride (DOTMA) 25 and other lipids of structure I defined in US-A-4,897,355, incorporated herein by reference or the ester analogues. The structure is as follows:



35 or an optical isomer thereof, wherein Y^1 and Y^2 are the same or different and are each $-\text{O}-$ or $\text{O}-\text{C}(\text{O})-$ wherein the carbonyl carbon is joined to R^1 of R^2 as the case may be; R^1 and R^2 are independently an alkyl, alkenyl, or alkynyl group of 6 to 24 carbon atoms, R^3 , R^4 and R^5 are 40

independently hydrogen, alkyl of 1 to 8 carbon atoms, aryl or aralkyl of 6 to 11 carbon atoms; alternatively two or three of R³, R⁴ and R⁵ are combined with the positively charged nitrogen atom to form a cyclic structure having 5 from 5 to 8 atoms, where, in addition to the positively charged nitrogen atom, the atoms in the structure are carbon atoms and can include one oxygen, nitrogen or sulfur atom; n is 1 to 8; and X is an anion.

Preferred embodiments are compositions wherein R¹ and 10 R² individually have from 0 to 6 sites of unsaturation, and have the structure



wherein the sum of a and c is from 1 to 23; and b is 0 to 6. Most preferably each of R¹ and R² is oleyl. 15 Particularly preferred embodiments are compositions wherein the long chain alkyl groups are fatty acids, that is, wherein Y¹ and Y² are alike and are —O—C(O)—.

Alternatively cationic lipids of the general structure I or the general structure II defined in US-A-5,459,127, 20 incorporated herein by reference may be used.

Other suitable cationic compounds are the non-lipid component stearylamine and 3β[N-(N'N'-dimethylaminoethane)-carbamyl] cholesterol (DC-Chol) (a lipidic component).

The liposomes, in addition to comprising cationic 25 components, generally also comprise non-ionic and/or zwitterionic components which include lipids, which may be phospholipids or other lipids not including phosphoryl groups. Preferably the lipids include phospholipids, such as natural or synthetic phosphatidylcholines, 30 phosphatidylethanolamines, phosphatidylserines in any of which the long chain alkyl groups (which may be joined through ester or ether linkages) may be saturated or unsaturated. Preferably the acyl groups of glyceride lipids are unsaturated. The components may include non- 35 lipidic components, for instance non-ionic surfactants such as sorbitan mono esters of fatty acids, and/or ethoxylated

fatty acids or other analogues, such as ethoxylated lanolins.

Best results are achieved when the liposomes include fusogenic lipids, which are usually phosphatidyl ethanolamines in which the acyl groups are unsaturated. Cholesterol may be included although it seems to render the liposomes too stable for adequate delivery of polynucleotide into target cells.

The amount of cationic component is preferably in the range 5 to 50% of the total moles of liposome forming components, preferably in the range 10 to 25% mole.

The liposome composition is generally in the form of an aqueous suspension for instance, a physiological buffer. Alternatively it could be a dried composition for rehydration.

The liposomes may be made by any of the generally used liposome forming techniques. The product liposomes may be multilamellar or unilamellar vesicles and may be relatively large (vesicle diameters in the range 300 nm to 2000 nm preferably with average diameters in the range 500-1000 nm), or small (vesicle diameters in the range 100 nm to 400 nm preferably with average diameters in the range 200 to 300 nm). Preferably the liposomes have a mean diameter not exceeding 500 nm, and preferably substantially all have diameters less than 2000 nm.

Preferably the liposomes are formed by a process in which the vesicles are formed, mixed with nucleotide to be entrapped and are then dehydrated, preferably by freeze drying, and subsequently rehydrated in aqueous composition to make dehydration-rehydration vesicles, and preferably subsequently subjected to micro fluidization to reduce the average size. Preferably the non-entrapped material is separated from liposomes by centrifugation or molecular sieve chromatography, after the rehydration and/or 35 microfluidization st ps.

The present inventor has established that the use of DRV's can provide increased entrapment levels for

polynucleotides. According to a first method aspect of the present invention there is provided a method of entrapping polynucleotide into liposomes involving the steps of:

- 5 1. forming an aqueous suspension comprising naked polynucleotide, which operatively encodes an immunogenic polypeptide useful to induce a desired immune response in a human or animal subject, and preformed liposomes,
- 10 2. freeze drying the suspension,
3. rehydrating the product of step 2,
4. subjecting the aqueous suspension of dehydration rehydration vesicles from step 3 to microfluidization; and
- 15 5. optionally separating non entrapped polynucleotide from liposomes.

According to a second method aspect of the invention there is provided a method of entrapping polynucleotide into liposomes involving the steps of:

- 20 1. forming an aqueous suspension comprising the naked polynucleotide and preformed cationic liposomes,
2. freeze drying the suspension,
3. rehydrating the product of step 2,
4. optionally subjecting the aqueous suspension of 25 DRV's from step 3 to micro fluidization; and
5. separating non entrapped polynucleotide from liposomes.

The dehydration-rehydration of both method aspects of the invention is substantially as described by Kirby and 30 Gregoriadis, 1984, the content of which is incorporated herein by reference. Thus, the liposomes in step 1 are small unilamellar (SUV's) and made in step 3 are preferably multilamellar liposomes (MLV's) respectively. The product liposomes of step 3 are generally called dehydration- 35 rehydration vesicles (DRV's). Micro fluidization of the DRV's is carried out substantially as described in WO-A-

92/04009, the disclosure of which is incorporated herein by reference and by Gregoriadis et al, 1990.

By using the DRV technique, the present inventor has established that an overall solute entrapment yield of 5 above 10% can be achieved. The inventor has established that up to 90% or even more of the polynucleotide present in the aqueous suspension subjected to the freeze drying step can be entrapped into the liposomes. Furthermore 10 micro fluidization, whilst resulting in a reduction of the percentage of polynucleotide incorporated, nevertheless allows entrapment rates for polynucleotide of more than 10%, for instance up to 50%, to be achieved. The level of 15 polynucleotide entrapment in the liposomal composition is preferably in the range 0.05 to 5, preferably 0.1 to 1.0, more preferably 0.2 to 0.5 $\mu\text{g}/\mu\text{ mole lipid}$ (or in the range 0.1 to 10 $\mu\text{g DNA per mg lipid}$).

This aspect of the invention is preferably used to make the liposomal preparations of the first two aspects.

In the second product and second method aspects of the 20 invention, although the polynucleotide is preferably one which operatively encodes for an immunogenic polypeptide useful to induce a desired immune response in a subject, it may also be useful for delivery of genes for other 25 applications, such as in gene replacement therapy, gene augmentation therapy, gene immunotherapy (for instance in cancer treatment), introduction of genes encoding therapeutically active polypeptides and introduction of genes encoding cell toxins (i.e. compounds which are toxic to cells, for instance for killing cancer cells).

30 The invention includes also the use of the novel liposomes or made by the novel processes of the invention in the manufacture of a composition for use in a method of therapy or prophylaxis. For instance the method may be the immunisation (vaccination) of a human or animal subject to 35 protect it against infection by infectious micro organisms. Alternatively an immune response may be generated by the gene product which is useful in immune therapy, for

instance to treat cancer. Alternatively the polynucleotide may be useful in gene augmentation or replacement therapy.

There is also provided in the present invention a new in vitro use of a novel composition according to the first 5 or second aspect or of the product of the method aspects of the invention in transfecting human or animal cells. The cells may subsequently be cultured in vitro and/or may be subsequently implanted into patient from which they were removed. Thus the invention includes an ex vivo 10 transplantation method, for instance of the type described in WO-A-93/14778, the content of which is incorporated herein by reference.

There is also provided a new pharmaceutical composition including a liposomal composition according to 15 the first aspect or the product of the second aspect of the invention and a pharmaceutically acceptable carrier. The composition may be suitable for administration by injection, for instance intravenously (i.v.), intramuscularly (i.m.), intraperitoneally (i.p.) orally or 20 subcutaneously (s.c.). Alternatively the composition is suitable for intranasal administration, or for delivery directly to the lung, by inhalation, or for transthermal delivery. Conventional pharmaceutical carriers used for liposomal administration can be used. The inventor has 25 found that the invention allows injection of the cDNA using im techniques whereas where naked DNA has been used as a vaccine in the past it has been found that special highly controlled injection protocols have to be followed to avoid any damage to the muscle and to inject in exactly the same 30 position to be able to provide reliable comparable results. For instance, it has been found that muscle regenerating agents must be preadministered to improve response.

There is also provided in the present invention a method of treatment in which the novel pharmaceutical 35 composition is administered to a human or animal subject. The method may thus be a method of inducing an immune response to an antigen encoded by the polynucleotide, for

instance which is an antigen of an infectious microbial agent. Alternatively the method may be for gene replacement or augmentation therapy, for immune therapy, for instance for cancer treatment or for the administration 5 of a polynucleotide encoding a pharmacologically useful polypeptide.

The present invention is illustrated in the following examples. In some of the examples DNA encoding luciferase is used as a model polynucleotide, luciferase being a model 10 gene product.

The drawings represent the results of some of the examples as follows:

Figure 1 is a series of bar charts showing the results mentioned in Example 2, table 4.

15 Figure 2 is a series of bar charts showing the results of example 3.

Figure 3 is a series of bar charts showing the results of example 4.

20 Figures 4 to 8 are a series of bar charts showing the results of example 5.

Example 1 - Entrapment and complexation of luciferase encoding DNA and in vitro transfection of cells

25 **Materials**

The sources and grades of egg phosphatidylcholine (PC), stearylamine (SA) and 1,2-bis (hexadecyloxy)-3-trimethylaminopropane (BisHOP) have been described elsewhere (Tan and Gregoriadis, 1989). N[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium (DOTMA) was a gift from GeneMedicine (Houston, Texas, USA). Phosphatidylserine (PS) and dioleoyl phosphatidylethanolamine (DOPE) were from Sigma Chemical Co. (Poole, Dorset, UK). The eukaryotic expression vector 30 pGL2-control ($\approx 3.99 \times 10^6$ Daltons) expressing the luciferase reporter gene from a SV40 promoter was purchased from Promega (Southampton, UK). The cationic 35

LipofectAMINE, obtained from Gibco BRL (Paisly, UK), was complexed with pGL2 in OptiMEM 1 reduced serum medium containing GLUTAMAX 1 (Gibco BRL) at a ratio of 15:1 (wt:st) before use. Deoxyribonuclease I (bovine pancreas, type II; specific activity: 2500 Kunitz units mg^{-1} protein) was from Sigma Chemical Co. RQ1 deoxyribonuclease (1 unit μl^{-1}) and the luciferase assay system kit were purchased from Promega. The pGL2 plasmid DNA was radiolabelled with ^{35}S -dATP (37 kBq; ICN Flow, Thame, UK) by the method of Wheeler and Coutelle (1995). All other reagents were of analytical grade.

Methods

Incorporation of plasmid DNA into liposomes

The dehydration-rehydration procedure (Kirby and Gregoriadis, 1984) was used for the incorporation of pGL2 plasmid DNA into liposomes. In short, 2 ml of small unilamellar vesicles (SUV) composed of PC (16 μmoles) and DOPE (molar ratio 1:1); PC (16 μmoles), DOPE and PS (molar ratios 1:1:0.5; negatively charged); PC (16 μmoles), DOPE and SA, or BisHOP (molar ratios 1:1:0.5; positively charged); PC (16 μmoles), DOPE and DOTMA (molar ratios 1:1:0.25; positively charged); and DOPE (16 μmoles) and DOTMA (molar ratio 1:0.25; positively charged) were prepared as described (Kirby and Gregoriadis, 1984), mixed with 10-100 μg (10-100 μl) pGL-2 into which tracer ^{35}S -labelled plasmid DNA pGL2 (6×10^4 - 7×10^4 dpm) had been added, and freeze-dried overnight. Following controlled (Kirby and Gregoriadis, 1984) rehydration and the generation of multilamellar (Gregoriadis et al, 1993) dehydration-rehydration vesicles (DRV), these were centrifuged at 40,000 xg for 25 min to remove non-incorporated DNA. The liposomal pellets were suspended in 0.1 M sodium phosphate buffer supplemented with 0.9% NaCl, pH 7.2 (PBS) and centrifuged again. The washed pellets were re-suspended in PBS and stored at 4°C until further use. In separate experiments, DNA-incorporating DRV as

above in mixture with free, non-incorporated DNA (ie. before centrifugation), were microfluidized (Gregoriadis et al, 1990) in a Microfluidizer M110S (Microfluidics, Newton, MA, USA) for 3 cycles or for 1, 2, 3, 5 and 10 cycles 5 (PC:DOPE-:DOTMA liposomes only). Separation of incorporated DNA from free DNA in microfluidized liposomes was carried out by centrifugation as above (1, 2, 3 and 5 cycles) or molecular sieve chromatography (10 cycles) using (Gregoriadis et al, 1990) a Sepharose 4B CL column 10 (Pharmacia). In some experiments, preformed (DNA-free) DRV were mixed with 10 or 50 µg DNA and either incubated at 20°C for 20 h or microfluidized for 3 cycles. In both cases, liposomes were centrifuged as above to separate adsorbed from non-adsorbed DNA. DNA incorporation into 15 liposomes or adsorption onto their surface was estimated on the basis of ³⁵S radioactivity recovered in the suspended pellets (non-microfluidized DRV and DRV microfluidized for 1,2,3 or 5 cycles) or the eluted fractions following chromatography (10 cycles).

20 Photon correlation spectroscopy

The z-average mean size of non-microfluidized and microfluidized DRV was measured in a Malvern Autosizer IIc as described elsewhere (Gregoriadis et al, 1993; Gregoriadis et al, 1990).

25 Incubation of liposomes with deoxyribonuclease

Non-microfluidized or microfluidized (3 cycles) DRV incorporating pGL2 plasmid DNA (0.75-22.5 µg) and tracer ³⁵S-labelled pGL2 plasmid DNA in 1 ml PBS were mixed with 100 units deoxyribonuclease I, and incubated at 37°C for 10 30 min. The reaction was stopped with 1 µl of 0.5 M EDTA (pH8) and the mixtures were centrifuged to separate digested from non-digested liposomal DNA. Digested DNA was estimated on the basis of released radioactivity in the supernatants. Preliminary work had established complete 35 degradation of 100 µg naked pGL2 under identical conditions. In other experiments, samples of similar liposomes containing 2 µg DNA were diluted to 100 µl with

a buffer containing 50 mM dithiothreitol and 50 µg/ml bovine serum albumin (fraction V; Sigma Chemical Co.), mixed with one unit of RQ1 deoxyribonuclease (Promega) and incubated at 37°C for 30 min. Digestion was terminated by 5 the addition of 1 µl 0.5 M EDTA (pH 8.0).

Agarose-gel electrophoresis

Samples of non-microfluidized or microfluidized DNA-incorporating DRV were incubated as above with or without RQ1 deoxyribonuclease and then extracted twice with a 10 phenol-chloroform mixture to remove lipid material. DNA in the aqueous layer was precipitated with ethanol, re-suspended in 20 µl TE buffer (10 mM Tris-Cl, pH 8.0 and 1 mM EDTA, pH 8.0) and subjected to agarose gel electrophoresis to determine DNA integrity.

15 Transfection experiments

Monkey kidneys COS-7 epithelial cells maintained in Dulbecco's modified Eagle medium (DMEM) with 200 mM FLUTAMAX I (Gibco BRL) containing 10% foetal calf serum, were harvested by trypsinization, seeded in 24-well plates 20 (Falcon) (5 X 10⁴ cells per well) and incubated for 18 to 24 h. Wells containing adherent cells at 70-80% confluency were washed with Dulbecco's phosphate buffered saline (without calcium or magnesium), pH7.2 (Gibco BRL) and then 25 transfected with 1 µg (6-18 µl) liposome-incorporated or LipofectAMINE (24µg lipid approximately) complexed with 1µg pGL2 DNA in a volume of 0.5 ml OptiMEM 1 reduced serum medium containing GLUTAMAX I. Following incubation at 37°C for four to six hours, the transfection medium was removed and replaced with 1 ml DMEM complete medium. Cells were 30 incubated for a total of 48h, lysed by scrapping into 200 µl of reporter lysis buffer (Promega) (cell lysis was enhanced by one cycle of freeze-thawing on dry ice) and then centrifuged at 12,000 xg for 5 min to obtain clear supernatants. These were assayed in triplicates for 35 luciferase activity with the luciferase assay system kit using an LKB 1251 luminometer with total light emission being recorded over 60 s. The protein concentration in

each of the lysates was measured by the method of Bradford (1976) using the Bio-Rad protein assay solution. Luciferase activity was expressed as relative light units per mg of protein (RLU/mg).

5 RESULTS

Incorporation of plasmid DNA into liposomes

Plasmid DNA was incorporated into neutral DRV (710-843 nm diameter; Examples 1.1. (1-6) composed of PC and DOPE, a phospholipid reputed (Legendre and Szoka, 1995) to facilitate transfection, and in similar liposomes supplemented with negatively (590-871 nm; Examples 1.2. (1-5) or positively (647-899 nm; Examples 1.3. (1-6), 1.4. (1-4) and 1.5 (1-2) charged amphiphiles. Charged vesicle bilayer surfaces are known (Bangham et al, 1974) to contribute to larger aqueous spaces in between bilayers and, thus, to greater solute entrapment. In the case of the negatively charged DNA, further improvement of incorporation in positively charged liposomes (cationic DRV) would be expected as a result of electrostatic interactions. Table 1 shows that incorporation of DNA in neutral DRV was considerable (44-55%), and in negatively charged DRV still more so (45-63%) for each of the amounts used (10-100 µg) (Examples 1.1.(1-6) and 1.2.(1-5)). Moreover, the possibility that most of the DNA was adsorbed onto the liposomal surface rather than incorporated within the vesicles, was thought unlikely: incubation of preformed DRV with naked DNA resulted in only a modest proportion (12-13%) of it being recovered with the DRV on centrifugation (Examples 1.1.7 and 1.2 (6-7)).

30 Microfluidization (3 cycles) of similar DNA-incorporating DRV in the presence of non-incorporated (free) DNA resulted in smaller (209-329 nm diameter) vesicles with a DNA content that was considerably reduced (to 10-20%) in the case of neutral DRV and to a lesser extent (to 37-51%) for negatively charged liposomes (Examples 1.1.(1-6) and 1.2.(1-5)). Again, very little (6 and 10%) DNA was recovered with liposomes when preformed DRV were

microfluidized in the presence of free DNA (Examples 1.1.7 and 1.2.(6-7)).

As anticipated, incorporation of DNA in cationic SA, BisHOP and DOTMA DRV was even greater (62-92%) with values 5 remaining high (50-83%) for microfluidized DRV (269-383 nm; Examples 1.3.(1-6), 1.4.(1-4) and 1.5.(1-2)). Here, however, after incubation or microfluidization of preformed cationic DRV (SA) with naked DNA, as much as 40-60% of the material used was recovered with the liposomes, presumably 10 as vesicle-surface bound (Examples 1.3(7-9)).

Incubation of liposomal DNA with deoxyribonuclease

Table 1 reveals that most of the DNA incorporated in neutral (45-72%), negatively charged (58-69%) or cationic (68-86%) liposomes was not degraded by DNase. In contrast, 15 recovery of DNA adsorbed to the surface of neutral or negatively charged after exposure to the enzyme was low (18%) (Examples 1.1.7 and 1.2.6). With DNA adsorbed to the surface of cationic (SA) liposomes, however, a considerable proportion (41-58%) of the latter was not available for 20 degradation by DNase (Examples 1.3.(7-8)). This may be attributed to a condensed DNA state known to occur with cationic vesicles and to be resistant to DNase (Legendre and Szoka, 1995). In view of these findings, the extent of 25 DNA incorporation within the cationic liposomes (as opposed to that bound to their surface) at the end of the incorporation procedure is difficult to estimate accurately.

Results of liposomal DNA vulnerability to DNase were 30 largely confirmed in experiments where samples of naked or liposomal DNA were exposed to RQ1 deoxyribonuclease and subsequently subjected to agarose gel electrophoresis. On the basis of intensity of staining and the appearance of smearing, it can be seen that, whereas naked plasmid DNA was completely digested, DNA entrapped within cationic 35 liposomes was fully protected. DNA in neutral and negatively charged DRV, on the other hand, was less well

protected as assessed by the lighter bands in the DNase digested samples compared to the undigested ones.

Transfection with liposomal pGL2 plasmid DNA

5 In experiments where COS-7 cells were transfected with pGL2 plasmid DNA incorporated in non-microfluidized DRV liposomes or complexed with LipofectAMINE, the latter serving as a control, significant levels of luciferase activity over background were observed with each of the DRV
10 formulations. However, levels of activity with cationic DRV (DOPE:DOTMA, PC:DOPE:DOTMA and PC:DOPE:SA) were approximately 10-fold higher than those achieved with neutral (PC:DOPE) and negatively charged (PC:DOPE:PS) and also the cationic PC:DOPE:BisHOP liposomes (Table 2). As
15 the size of liposomes may be related to the efficiency of transfection, related experiments were also carried out with DNA incorporated in DRV which were microfluidized for 1,2,3,5 or 10 cycles to produce vesicles of progressively smaller size (386, 319, 262, 235 and 123 nm z-average
20 diameter respectively; not shown). Table 2 indicates that microfluidization (3 cycles) of the PC:DOPE:DOTMA DRV improved their transfection efficiency by 10-fold. However, transfection experiments with PC:DOPE:DOTMA, DOPE:DOTMA, PC:DOPE and PE:DOPE:PS liposomes subjected to
25 5 or 10 cycles of microfluidization failed to show significant luciferase activity (results not shown), which can be explained by the microfluidization-induced progressive smearing of DNA.

30 It appears that of all the DRV preparations tested, positively charged SA and DOTMA DRV were more efficient than the remainder, with the microfluidized preparation (DOTMA) exhibiting the highest values of transfection. However, even this preparation was 10-15 fold less efficient than the control LipofectAMINE.

TABLE 1

Example	Liposomes	'E' ntrapped or 'C'omplexed	DNA used (μ g)	% incorporated DNA (% retained DNA)	
				DRV	Microfluidized DRV
1.1.1	PC:DOPE	E	10	40.4 (71.9)	12.5 (49.8)
1.1.2	PC:DOPE	E	20	43.8 (62.0)	17.3 (48.1)
1.1.3	PC:DOPE	E	25	41.1 (67.1)	16.9 (48.0)
1.1.4	PC:DOPE	E	30	39.5 (60.0)	20.6 (42.4)
1.1.5	PC:DOPE	E	50	41.3 (45.3)	10.1 (44.0)
1.1.6	PC:DOPE	E	100	55.4	-
1.1.7	PC:DOPE	C	10	12.1 (17.8)	6.8 (10.2)
1.2.1	PC:DOPE:PS	E	10	55.8 (69.1)	44.6 (67.8)
1.2.2	PC:DOPE:PS	E	20	61.2 (60.9)	40.9 (66.1)
1.2.3	PC:DOPE:PS	E	25	61.0 (58.2)	42.3 (62.6)
1.2.4	PC:DOPE:PS	E	50	45.5 (58.0)	37.0 (67.1)
1.2.5	PC:DOPE:PS	E	100	63.0	51.0
1.2.6	PC:DOPE:PS	C	10	12.0 (17.6)	10.2 (9.8)
1.2.7	PC:DOPE:PS	C	50	13.3	10.8
1.3.1	PC:DOPE:SA	E	10	64.1 (78.1)	50.3 (63.1)
1.3.2	PC:DOPE:SA	E	20	71.9 (75.0)	65.5 (59.9)
1.3.3	PC:DOPE:SA	E	25	82.3 (75.2)	64.7 (58.2)
1.3.4	PC:DOPE:SA	E	30	74.8 (70.1)	56.2 (58.0)
1.3.5	PC:DOPE:SA	E	50	71.2 (67.9)	50.9 (57.4)
1.3.6	PC:DOPE:SA	E	100	84.4	60.1
1.3.7	PC:DOPE:SA	C	10	59.7 (41.1)	31.6 (40.2)
1.3.8	PC:DOPE:SA	C	25	45.1 (58.5)	12.9 (41.0)
1.3.9	PC:DOPE:SA	C	50	40.3	16.3
1.4.1	PC:DOPE:BH	E	10	68.4 (75.3)	51.9 (63.1)
1.4.2	PC:DOPE:BH	E	20	70.8 (70.2)	55.5 (64.0)

1.4.3	PC:DOPE:BH	E	25	62.3 (69.9)	52.2 (78.2)
1.4.4	PC:DOPE:BH	E	50	75.6	62.1
1.5.1	PC:DOPE: DOTMA	E	50	81.0 (85.9)	76.3 (79.1)
1.5.2	PC:DOPE: DOTMA	E	100	92.6	83.2

BH = Bishop

TABLE 2
Luciferase activity in transfected cells

Example	Liposomes	Luciferase activity RLU/mg
1.6.1	DOPE:DOTMA	1.5 x 10 ⁴
1.6.2	PC:DOPE:DOTMA	1.3 x 10 ⁴
1.6.3	PC:DOPE:DOTMA Mfx3	7 x 10 ⁴
1.6.4	PC:DOPE:BisHOP	2 x 10 ³
1.6.5	PC:DOPE:SA	9 x 10 ³
1.6.6	PC:DOPE	2 x 10 ³
1.6.7	PC:DOPE:PS	3 x 10 ³
1.6.8	LIPOFECTAMINE	3 x 10 ⁶

Mf x 3 - microfluidized, 3 cycles.

Example 2 - Immune response after in vivo transfection

Using the materials as described and from the sources
 5 of Example 1 (and in addition 3 β [N-(N'N'-dimethylaminoethane)-carbamyl] cholesterol, DC-CHOL, obtained from Dr C Kirby and DOTAP - 1,2-dioleyloxy-3-trimethylammonium propane) experiments were conducted to determine the immune response after in vivo transfection.
 10 The polynucleotide is plasmid DNA expressing the Hepatitis B surface antigen (S region, plasmid pRc/CMV-HBS of the ayw type (Davis HL et al)). Liposomes were formed in each case using 16 micromoles PC (12mg) throughout, with the cationic lipid specified in the tables in the ratios used in the
 15 tables. Plasmid DNA was either entrapped into the liposomes (in the amount specified in the table) using the methods of example 1, or complexes of preformed cationic liposomes and DNA were made, by mixing those components together in aqueous suspension (using techniques comparable
 20 to the prior art by Eppstein, mentioned above).

Liposomes with entrapped DNA, the complexes and naked DNA were then administered to mice for the in vivo transfection experiments. Balb/c mice, in groups of three or four, were injected intramuscularly (hind leg) with the preparations in an amount such as to administer μ g DNA
 25

tables. Plasmid DNA was either entrapped into the liposomes (in the amount specified in the table) using the methods of example 1, or complexes or preformed cationic liposomes and DNA, by mixing those components together in aqueous suspension (using techniques comparable to the prior art by Eppstein, mentioned above).

Liposomes with entrapped DNA, the complexes and naked DNA were then administered to mice for the *in vivo* transfection experiments. Balb/c mice, in groups of three or four, were injected intramuscularly (hind leg) with the preparations in an amount such as to administer the level of DNA specified in table 4. For each test, the amount of lipid in the liposome preparation administered to the mice is approximately constant and is a value in the range 1-2 mg total PC lipid.

Subsequently mice were bled and the sera were tested by ELISA techniques to determine the immune response. In these *in vivo* experiments, the ELISA test is carried out as described by Davis et al (1987) using the S region antigen of ayw Type Hepatitis B. Tests were used to determine anti-HBS Ag (S region ayw type) - IgG₁, IgG_{2a} and IgG_{2b}. The immune responses obtained are expressed as log₁₀ (mean + or - standard deviation) of serum dilutions required to give an absorbance reading (in the horseradish peroxidase ELISA test) of about 0.200.

In the experiments the results of which are reported in table 4, the mice were injected on days 0, 10, 20, 27 and 37 and were bled on days 26, 34 and 44. In the results given in table 5, the mice were injected on days 0, 7, 14, 21 and 28 and were bled on days 21 and 28.

RESULTS AND CONCLUSIONS

TABLE 3

Entrapment of plasmid DNA (into liposomes).

5	Example	Liposomes (molar ratio)	Entrapped or complexed	DNA used (μ g)	Entrapment/ complexation rate (% of DNA used)
10	2.1	PC, DOPE (1:0.5)	E	100	57.3
			E	150	53.6
15	2.2	PC, DOPE, DC-CHOL (1:0.5:0.25)	E	100	95.4
			E	150	78.9
10	2.3	PC, DOPE, DOTAP (1:0.5:0.25)	E	100	82.9
			E	100	78.4
			E	150	77.1
			E	150	82.1
15	2.4	PC, DOPE, DOTAP (1:0.5:0.25)	C	100	93.9
			C	150	83.3

TABLE 4
 Immune response * (ELISA results \pm SD) of mice immunised with free or liposome entrapped DNA.

Lipids, ratios preparation (example)	Injected DNA (ug)	26 days			34 days			44 days		
		IgG ₁	IgG _{2a}	IgG ₁						
PC, DOPE, DOTAP (2:3)	5	3.1 \pm 0.2	2.2	ND	4.2 \pm 0.4	3.2 \pm 0.0	3.1 \pm 0.2	5.5 \pm 0.2	3.1 \pm 0.3	2.7 \pm 0.0
(1.0:0.5:0.25)	10	3.2 \pm 0.0	ND	ND	4.2 \pm 0.0	3.2 \pm 0.0	3.2 \pm 0.0	4.8 \pm 0.5	3.4 \pm 0.3	3.0 \pm 0.3
PC:DOPE: DC-CHOL (2:2)	5	3.0 \pm 0.3	2.2	ND	4.0 \pm 0.2	3.0 \pm 0.3	2.7 \pm 0.0	5.2 \pm 0.2	3.0 \pm 0.3	2.9 \pm 0.3
(1.0:0.5:0.25)	10	3.0 \pm 0.3	ND	ND	4.0 \pm 0.2	3.6 \pm 0.2	3.1 \pm 0.2	5.0 \pm 0.2	3.4 \pm 0.3	3.2 \pm 0.0
Naked DNA	5	2.2 \pm 0.0	ND	ND	2.2 \pm 0.0	2.2 \pm 0.0	1.8 \pm 0.0	3.2 \pm 0.0	2.2 \pm 0.0	2.2 \pm 0.0
	10	2.4 \pm 0.2	ND	ND	2.2 \pm 0.0	2.2 \pm 0.0	1.8 \pm 0.0	2.9 \pm 0.3	2.2 \pm 0.0	2.2 \pm 0.0

ND means "not determined"

* log 10 of dilutions needed to give a reading of about 0.200 in the ELISA test.

See also Figure 1 in which A represents the results of the liposomes including DOTAP, B the results of liposomes including DC-Chol, C the results of liposomes including stearylamine in the same amount (not shown in the tabl) and D naked DNA, in each case for the case where 5 μ g DNA is administered. White bars are IgG₁ values, black bars are IgG_{2a} and dotted bars are IgG_{2b}.

TABLE 5

Immune response (ELISA results \pm SD) of mice immunised with naked, complexed and entrapped DNA

DNA preparation (example)	Test Report	Injected DNA (ug)	21 days		28 days	
			IgG ₁	P	IgG ₁	P
PC, DOPE, DOTAP 1.0:0.5:0.25 (entrapped) (2.3)	a	1	2.2 \pm 0.0		2.5 \pm 0.3	a vs g <0.05
	b	10	3.2 \pm 0.0	b vs a,c,d,e,f,g,h <0.0001	4.0 \pm 0.2	b vs h <0.0007
PC, DOPE DOTAP 1.0:0.5:0.25 (complexed) (2.4)	c	1	2.2 \pm 0.0		2.4 \pm 0.2	
	d	10	2.2 \pm 0.0		2.8 \pm 0.2	d vs b <0.0032
PC, DOPE (2.1) 1.0:0.5	e	1	2.2 \pm 0.0		2.2 \pm 0.0	
	f	10	2.2 \pm 0.0		2.7 \pm 0.0	f vs b <0.001 f vs h <0.003
Naked DNA	g	1	2.2 \pm 0.0		2.2 \pm 0.0	
	h	10	2.2 \pm 0.0		2.2 \pm 0.0	h vs d <0.0001

In the tables the columns P show the result of students paired t-test indicating the confidence level that the results, as specified, are different from one another.

Table 3 shows that the percentage entrapment is extremely high for lipid compositions containing cationic lipids. The complexation rate of plasmid DNA with preformed lipids is also very high. In each case, the percentage entrapment/complexation rate is little effected by the use of 100 μ g or 150 μ g of DNA.

Table 4 and Figure 1 show that the immune response following immunisation of mice with entrapped DNA encoding Hepatitis B surface antigen is much higher than following immunisation with naked DNA. Whilst this effect is already apparent after 26 days from the start of the experiment,

the effect becomes yet more pronounced as the experiment continues. The effect is particularly pronounced for IgG₁ although the levels of both IgG_{2a} and IgG_{2b} are also present in increased amounts as compared to naked DNA transmission, 5 after all bleeds.

The results in table 5 show that for naked DNA, no response is seen even after 28 days following the first administration of DNA.

For DNA entrapped within neutral liposomes (PC, DOPE) 10 there is no increase in immune response at 21 days although there is slight increase in response for the higher amount of injected DNA after 28 days and this is significantly higher than the response after administration of when naked DNA.

15 For the complex of preformed cationic liposomes and DNA, the immune response does appear to be developing after 28 days from the start of the experiment, for both levels of DNA administration and is significantly higher than the response after administration of naked DNA. However the 20 immune responses are not as high as those obtained for the DNA entrapped within cationic liposomes.

For cationic liposomes with entrapped DNA after 21 days, already the immune response is significantly higher than all of the other examples, where the amount of DNA 25 injected is 10 μ g; though there is no significant difference between the response after administration of 1 μ g DNA entrapped in cationic liposomes and any of the other examples in which 1 μ g DNA is administered. After 28 days, with the lower level of DNA administered (1 μ g) there is a 30 significantly increased immune response as compared to high or low amounts of naked DNA. However there is no significant difference to the response following administration of the lower levels of DNA complexed with cationic liposomes or entrapped in neutral liposomes. For 35 the higher level of DNA administered (10 μ g) entrapped in cationic liposomes, after 28 days the immune response is significantly higher than all of the other tests.

Example 3 - Cytokine levels in the spleens of mice immunized with naked, complexed or liposomal entrapped plasmid DNA

5 Balb/c mice in groups of four (the same protocol and experiment as example 2) were injected intramuscularly on days 0.7, 14, 21 and 28 with 1 (white bars) or 10 μ g (black bars) of pRc/CMV HBS entrapped in positively charged liposomes composed of PC, DOPE and DOTAP (A), uncharged liposomes composed of PC and DOPE (B), complexed with 10 similar performed cationic DOTAP liposomes (C) or in naked form (D). "Control" represents cytokine levels in normal 15 unimmunized mice. Three weeks after the final injection, mice were killed and their spleens subjected to cytokine analysis. Endogenous levels of IFN- γ and IL-4 in the 20 spleen were determined by the method of Nakane et al as previously modified by de Souza et al. Individual spleens were weighed, homogenized in ice-cold RPMI containing 1% 3-[(cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS; Sigma) in a Dounce tissue homogenizer and 10% (wt/vol) homogenates were prepared. Homogenates were left 25 on ice for 1 h and insoluble debris were then removed by centrifugation at 2000 Xg for 20 min. The clear supernatants were stored at -70°C.

25 **Cytokine assays**

Standard capture ELISAs were used with monoclonal antibody pairs and Maxisorp (NUNC, UK) plates. Primary 30 monoclonal antibodies against IFN- γ (R46A2) and IL-4 (11B11) and secondary biotinylated anti-mouse IL-4 (BVD6-24G2) and anti-mouse IFN- γ (XMG1.2) monoclonal antibodies (Pharmingen, USA) were used with streptavidin peroxidase (Dako, Denmark) and o-phenylenediamine (Sigma) as substrate. Recombinant IFN- γ and IL-4 standards were from Pharmingen. Results (mean \pm SE) are expressed as ng/spleen 35 from at least 4 mice. The results are shown in Figure 2 in which each bar represents the mean \pm SE of a group of 4 mice (a-d representing the liposomes as specified above).

The data (Fig. 2) show that activation for both Th1 and Th2 subsets was greater with liposome-entrapped DNA when compared with naked or complexed DNA. This finding was also confirmed in preliminary T cell proliferation assays against the HBsAg antigen *in vitro*. It therefore appears that immunization with liposome-entrapped plasmid DNA induces both humoral and cell-mediated immunity.

Example 4 - Immune responses in mice after a single injection of plasmid DNA

Most reports on naked DNA vaccination have employed protocols of multiple injections but a single dose also produces a humoral response to the encoded antigen (Davis et al Human Gene Therapy 1993, and Raz et al PNAS 1994). For instance, total IgG response for the naked pRc/CMV HBS (identical to the plasmid used here) was detectable 1-2 weeks after injection, to reach peak values by 4-8 weeks (Davis et al 1996).

Balb/c mice in groups of four were injected once intramuscularly with 2 (white bars see figure 3) or 10 μ g (black bars) of pRc/CMV HBS entrapped in positively charged liposomes composed of PC, DOPE and DOTAP (A), uncharged liposomes composed of PC and DOPE (B), complexed with preformed similar DOTAP liposomes (C) or in the naked form (D). Anti-HBsAg IgG₁ responses were analysed (ELISA) in sera obtained at time intervals after injection. Immune responses were mounted by all mice injected with liposomal DNA but became measurable only at 20-27 days. The remaining details were as in example 2. Differences in log₁₀ values (both doses; all time intervals) between mice immunized with cationic liposomal DNA and mice immunized with naked DNA were statistically significant (P<0.0001-0.002). In a fifth group of four mice immunized once as above with 10 μ g pRc/CMV HBS entrapped in anionic liposomes composed of PC, DOPE and PS (made by the method as described in Example 1), IgG₁ responses (1 g₁₀) were 2.25 \pm 0.0 and 2.73 \pm 0.0 at 21 and 29 days resp ctiv ly.

Under the present conditions of single immunization (Fig. 3) with much lower doses of pRc/CMV HBS (2 and 10 μ g), anti-HBsAg IgG, response for naked and complexed DNA was barely detectable even by seven weeks. In contrast, there 5 was an early and pronounced IgG, response for DNA entrapped in cationic liposomes and a delayed but significant response for DNA entrapped in neutral or negatively charged liposomes (Fig. 3).

10 **Example 5 - Humoral and cell-mediated response of in-bred and out-bred mice injected with Hep B antigen in cationic liposomes**

Groups of mice (4-5 animals per group) were injected (i.m., i.p., i.v. or s.c.) twice (on days 0 and 7) with 10 μ g pRc/CMV HBS (encoding the S region of hepatitis B 15 surface antigen; subtype ayw) entrapped in cationic DRV liposomes composed of egg phosphatidylcholine (PC), dioleoyl phosphatidylcholine (DOPE) and 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP) (molar ratios 1:5.5:0.25) (produced using the general techniques and 20 materials described above in example 1), or with 10 μ g of naked pRc/CMV HBS (both in PBS). Animals were bled at time intervals and IgG₁, IgG_{2a} and IgG_{2b} were measured by ELISA in the plasma. At the end of the experiment (38 days after 25 the first injection) animals were killed and the cytokines IFN γ and IL-4 were measured in the spleen as described (for additional experimental details see Gregoriadis *et al*, 1997). The cytokines were also measured in the spleen of control (intact) mice.

Results

30 Results show that immune responses (mean \pm SD) for both strains of mice became measurable only after 21 (IgG₁; Fig 4) or 28 days (IgG_{2a} and IgG_{2b}; Figs 5 and 6 respectively) after the first injection. Responses for liposomal DNA (black bars) were generally significantly greater than 35 those for naked DNA (dotted bars) (both strains and all routes, specially im, sc and iv). Significant levels ($P<0.05$) increase in the order of + *, **, ***.

Analysis of the cytokines IFN γ (Th1 response) (Fig. 7) and interleukin 4 (IL-4) (Th2 response) (Fig. 8) revealed significantly greater levels for liposomal DNA by the im and sc routes in both strains for the (iv route in T/o mice). There was no difference in values between liposomal and naked DNA for the ip route (both strains).

Example 6 - Entrapment value for six different plasmid DNA's

³⁵S-labelled plasmid DNA (10-500 μ g) was incorporated 10 into or mixed with neutral, anionic or cationic dehydration rehydration vesicle (DRV) using the technique described in example 1. The plasmid DNAs used were the following:

pGL2 - encoding luciferase, as for example 1
pRc/CMV HBS - hepatitis B surface antigen (S) region 15 as in example 2
pRSVGH - encoding human growth hormone, a therapeutic protein
pCMV4.65 - micobacterium leprosy protein, an antigen
pCMV4.EGFP - "fluorescent green protein"
20 VR1020 - schistosome protein, an antigen.

The lipids used included neutral lipids PC and DOPE described in example 1 above, anionic lipids, PS, phosphatidyl serine, described in example 1 or phosphatidyl glycerol (PG) and cationic compounds stearylamine (SA), 25 BisHOP and DOTMA, all described in example 1, DC-Chol and DOTAP, as used in example 2 and, in addition 1,2-dioleoyl-3-dimethylammonium propane DODAP.

The table below indicates whether the plasmid DNA was incorporated (that is encapsulated into (a) or merely 30 admixed (b) with the DRV). The table further indicates the lipid components and the incorporation values for DNA. Previous tests had shown that incorporation values using different amounts of DNA for each of the DRV formulations did not differ significantly. Results were therefore 35 pooled and the values shown in the table are means of values obtained from 3 to 5 experiments.

Table 6. Incorporation of plasmid DNA into liposomes

5	Liposomes	Incorporated plasmid DNA (% of used)					
		pGL2	pRc/CMV HBS	pRSVGH	pCMV4.65	pCMV4. EGFP	VR1020
10	PC, DOPE ^a	44.2	55.4	45.6	28.6		
	PC, DOPE ^b	12.1		11.3			
	PC, DOPE, PS ^a	57.3					
	PC, DOPE, PS ^b	12.6					
15	PC, DOPE, PG ^a			53.5			
	PC, DOPE, PG ^b			10.2			
	PC, DOPE, SA ^a	74.8					
	PC, DOPE, SA ^b	48.3					
20	PC, DOPE, BisHOP ^a	69.3					
	PC, DOPE, DOTMA ^a	86.8					
	PC, DOPE, DC-Chol ^a		87.1	76.9			
	PC, DOPE, DC-Chol ^b			77.2			
25	PC, DOPE, DOTAP ^a		80.1	79.8	52.7	71.9	89.6
	PC, DOPE, DOTAP ^b		88.6	80.6	67.7		81.6
	PC, DOPE, DODAP ^a			57.4			
	PC, DOPE, DODAP ^b			64.8			

30

The results show that, by encapsulating the DNA, far higher values for the level of incorporation can be achieved where the lipid is negatively charged. Where cationic lipid is used, the incorporation values do not appear to differ significantly between encapsulation and physical admixture, although it seems that encapsulation gives high r values

where the cationic component is a non-lipidic compound (stearylamine).

Example 7 - The effective lipid composition of cationic liposomes on the immune response to the HBsAg antigen encoded by the entrapped pRc/CMV HBS

Using the general techniques described above, plasmid DNA was entrapped into various cationic liposomes. The lipids used and the molar ratios are shown in table 7 below. The lipids were as used in previous examples and additional PE is phosphatidyl ethanolamine with egg lipid, and DSPC, di-stearoylphosphatidylcholine, a saturated lipid. Balb/c mice were injected intramuscularly in groups of 5 with 10 μ g of free or liposome entrapped plasmid on day zero, two weeks and five weeks. Animals were bled at 8, 10 and 13 weeks and sera assayed by ELISA for anti-HBsAg (S region) IgG, antibodies. The technique used is as generally described in example 5. The results are shown in Table 7 below.

TABLE 7

Liposomes mole ratios of lipids	IgG, response (\log_{10} reciprocal end point dilution \pm SD)		
	8 weeks	10 weeks	13 weeks
A. PC:DOPE:DOTAP (1:0.5:0.25)	2.99 \pm 0.24	2.87 \pm 0.29	2.63 \pm 0.15
B. PC:PE:DOTAP (1:0.5:0.25)	2.99 \pm 0.56	3.17 \pm 0.64	2.75 \pm 0.35
C. PC:DOTAP (1:0.25)	2.05 \pm 0.66	1.98 \pm 0.58	1.83 \pm 0.33
D. DSPC:DOPE:DOTAP (1:0.50:0.25)	2.51 \pm 0.19	2.48 \pm 0.19	1.90 \pm 0.00
E. PC:Chol:DOTAP (1:0.50:0.25)	2.57 \pm 0.35	2.51 \pm 0.27	2.14 \pm 0.23
F. Free pRc/CMV HBS	1.30 \pm 0.00	1.30 \pm 0.00	1.23 \pm 0.13

Statistical analysis of the results (unpaired T-test) revealed significant differences between (a) all liposomal DNA formulations and free DNA ($P<0.0001-0.0441$; all time intervals), PC:DOPE:DOTAP and PC:DOTAP ($P<0.0036-0.0357$; all

time intervals), PC:PE:DOTAP and PC:DOTAP ($P<0.0095-0.0385$;
10 and 13 weeks), PC:DOPE:DOTAP and
DSPC:DOPE:DOTAP ($P<0.0001-0.0138$; 8 and 13 weeks) and
PC:DOPE:DOTAP and PC:CHOL:DOTAP ($P<0.0158$; 13 weeks).

5 Results suggest that in terms of liposomal efficacy in
promoting immune responses, (a) DOPE can be replaced by PE
without loss of liposomal efficacy (DOPE and PE are both
unsaturated lipids), b) phosphatidylethanolamine (PE or
10 DOPE) renders liposomes more efficient than liposomes
without this type of lipid, c) replacement of PC with the
saturated DSPC reduces liposomal efficacy, d) liposomes
with cholesterol but without phosphatidylethanolamine are
nearly as effective as liposomes with
phosphatidylethanolamine but only at 8 and 10 weeks.

15 **Example 8 - Entrapment of pRc/CMV HBS into non-phospholipid liposomes**

20 In this example various liposome forming components
other than phospholipids were used to entrap the hepatitis
antigen used in previous examples. The liposome forming
components included a glyceride, MonoPal; 1-Monopalmitoyl-
rac-glycerol, and nonionic surfactants, Span 60(-sorbitan
25 monostearate) and Solulan 24, (a 24 mole ethoxylated
complex of lanolin alcohols and related fatty alcohols).
(Span 60 and Solulan 24 are trademarks). The mole ratios
of the liposome forming components are shown in the table.
The other components are as used in the above examples.

30 The results show that adequate entrapment rates can be
achieved using liposome forming components not including
phospholipids. Nonionic surfactants, optionally in
combination with other non phospholipid lipidic components
such as cholesterol can give adequate entrapment rates. It
was noted that liposomes formed in examples 8.4, 8.5, 8.6
and 8.8 precipitated on standing and were thus not
optimised in terms of composition.

35 The entrapment rates are shown in Table 8 below.

TABLE 8

Exempl	Liposomes	Molar Ratios (also absolute in terms μ mole)	Entrapm nt (% of DNA used)	
8.1	MonoPal:Chol:DOTAP	16:16:4	63.2	
8.2	MonoPal:Chol:DOTAP	20:8:4	84.0	
5	8.3	MonoPal:Chol:DOTAP	16:8:4	64.1
8.4	MonoPal:DOPE:DOTAP	16:8:4	28.1	
8.5	MonoPal:Chol:DOPE: DOTAP	16:16:8:4	46.4	
8.6	SPAN60:Chol:DOTAP	16:16:4	83.6	
8.7	SPAN60:Solulan: Chol:DOTAP	16:0:72:16:4	66.0	
10	8.8	SPAN60:Chol:DOTAP	20:8:4	55.8

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CLAIMS

1. A composition comprising liposomes formed from liposome forming components and, entrapped within the liposomes, a polynucleotide operatively coding for an immunogenic polypeptide useful to induce a desired immune response in a human or animal subject.

5

2. A composition according to claim 1 in which the polynucleotide is double stranded DNA.

10

3. A composition according to claim 2 in which the polynucleotide is in the form of a plasmid including promoter and, optionally, ribosome binding sequences.

15

4. A composition according to claim 1 in which the polynucleotide is RNA, preferably mRNA.

5. A composition according to any preceding claim in which the immunogenic polypeptide comprises an antigen or fragment of an antigen of an infectious microbe.

20

6. A composition according to any preceding claim in which the liposome forming components are selected such that the liposomes have no overall charge.

7. A composition according to any of claims 1 to 5 in which the liposome forming components include at least one cationically charged liposome forming component, in an amount such that the liposome forming components have an overall cationic charge.

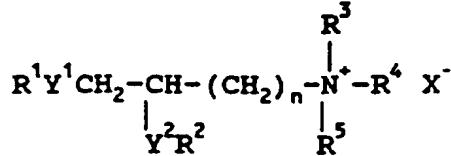
25

8. A composition comprising liposomes formed from liposome forming components including at least one cationically charged component in an amount whereby the liposome forming components have an overall positive charge and polynucleotide encoding a desired polypeptide product and is characterised in that the polynucleotide is entrapped within the liposome.

30

9. A composition according to claim 7 or claim 8 in which the cationic component is a glyceride having the general formula

35



or an optical isomer thereof, wherein Y¹ and Y² are the same or different and are each -O- or O-C(O)- wherein the carbonyl carbon is joined to R¹ of R² as the case may be; R¹ and R² are independently an alkyl, alkenyl, or alkynyl 5 group of 6 to 24 carbon atoms, R³, R⁴ and R⁵ are independently hydrogen, alkyl of 1 to 8 carbon atoms, aryl or aralkyl of 6 to 11 carbon atoms; alternatively two or three of R³, R⁴ and R⁵ are combined with the positively charged nitrogen atom to form a cyclic structure having 10 from 5 to 8 atoms, where, in addition to the positively charged nitrogen atom, the atoms in the structure are carbon atoms and can include one oxygen, nitrogen or sulfur atom; n is 1 to 8; and X is an anion.

10. A composition according to claim 9 in which R¹ and R² individually have from 0 to 6 sites of unsaturation, 15 and have the structure



wherein the sum of a and c is from 1 to 23; and b is 0 to 6.

11. A composition according to claim 7 or claim 8 in which the cationic component is selected from DOTAP, BisHOP, DC-Chol and stearylamine.

12. A composition according to any preceding claim in which the liposome forming components include a fusogenic 25 component, preferably a phosphatidyl ethanolamine.

13. A composition according to any preceding claim in which the liposomes have been formed by a dehydration-rehydration process, preferably followed by a micro fluidization step or extrusion.

14. A composition according to any preceding claim in which the mean diameter of the liposomes is in the range 30 100 to 1000 nm, preferably in the range 200 - 500 nm.

15. A composition according to any preceding claim comprising 0.1 to 10 μg of polynucleotide per mg liposome 35 forming components.

16. Use of liposomes as defined in any preceding claim in the manufacture of a composition for use in a

method of treatment of a human or animal by therapy or prophylaxis.

17. Use according to claim 16 in which the method is vaccination to immunise against infective microbes or a 5 cancer cell.

18. Use according to claim 16 or claim 17 in which the composition is administered by intramuscularly.

19. A pharmaceutical composition comprising a 10 liposome preparation according to any of claims 1 to 15 and a pharmaceutically acceptable excipient.

20. A method of treatment of a human or animal subject in need of such treatment in which a pharmaceutical composition according to claim 19 is administered to the human or animal subject whereby the polypeptide product of 15 the polynucleotide is expressed in target cells.

21. A method according to claim 20 in which the pharmaceutical composition is administered intramuscularly.

22. A method according to claim 20 in which the pharmaceutical composition is administered subcutaneously.

23. A method according to claim 20 in which the pharmaceutical composition is administered intravenously.

24. A method according to claim 20 in which the pharmaceutical composition is administered intraperitoneally.

25. A method of transfection of cells removed from a human or animal subject in which the cells are contacted with a liposomal composition according to any of claims 1 to 15, and the cells are cultured, whereby transfection of the cells and expression of the polynucleotide and 30 synthesis of the polypeptide product of the polynucleotide take place.

26. A method according to claim 25 in which the cultured cells are subsequently implanted into the host animal or human, whereby expression of the polypeptide 35 takes place in vivo.

27. A method according to any of claims 20 to 24 in which the expressed polynucleotide induces a desired immune

response, preferably a response to an antigen of an infectious microbe or a cancer cell.

28. A method of entrapping polynucleotide into liposomes involving the steps of:

5 1. forming an aqueous suspension comprising naked polynucleotide, which operatively encodes an immunogenic polypeptide useful to induce a desired immune response in a human or animal subject, and preformed liposomes,

10 2. freeze drying the suspension,

15 3. rehydrating the product of step 2,

15 4. subjecting the aqueous suspension of dehydration rehydration vesicles from step 3 to microfluidization; and

15 5. optionally separating non entrapped polynucleotide from liposomes.

29. A method according to claim 28 in which the liposomes are cationic liposomes.

30. A method of entrapping polynucleotide into liposomes involving the steps of:

20 1. forming an aqueous suspension comprising the naked polynucleotide and preformed cationic liposomes,

25 2. freeze drying the suspension,

25 3. rehydrating the product of step 2,

25 4. subjecting the aqueous suspension of dehydration rehydration vesicles from step 3 to microfluidization; and

25 5. optionally separating non entrapped polynucleotide from liposomes.

30 31. A method according to any of claims 28 to 30 in which the entrapment rate of polynucleotide in steps 1-4 is in the range 10 to 90%, preferably in the range 20 to 85% of polynucleotide in the suspension in step 1.

35 32. A method according to any of claims 28 to 31 in which, in the final product the level of polynucleotide is in the range 0.1 to 20 μ g/mg liposome forming components.

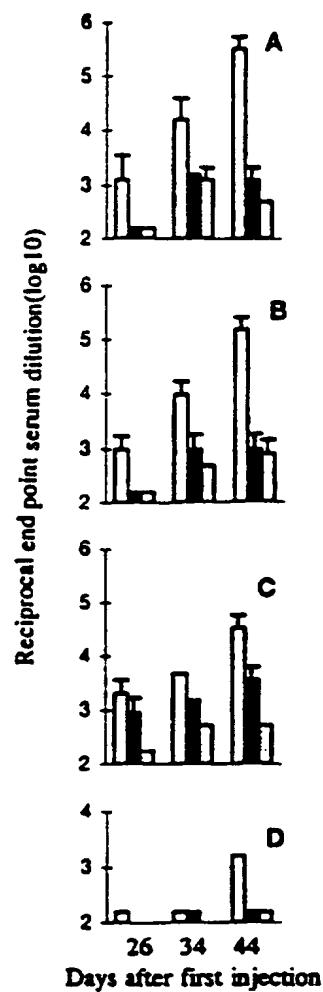


Figure 1

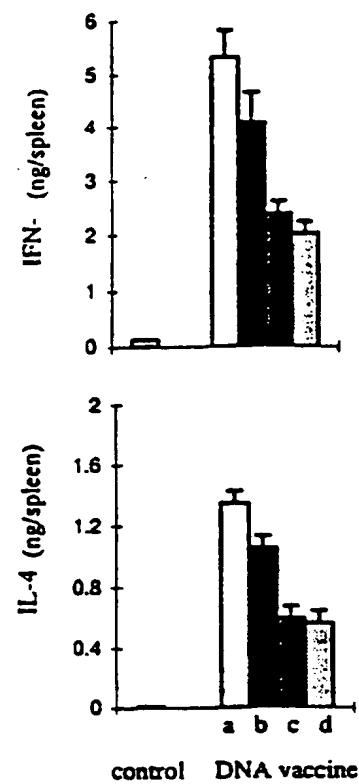


Figure 2

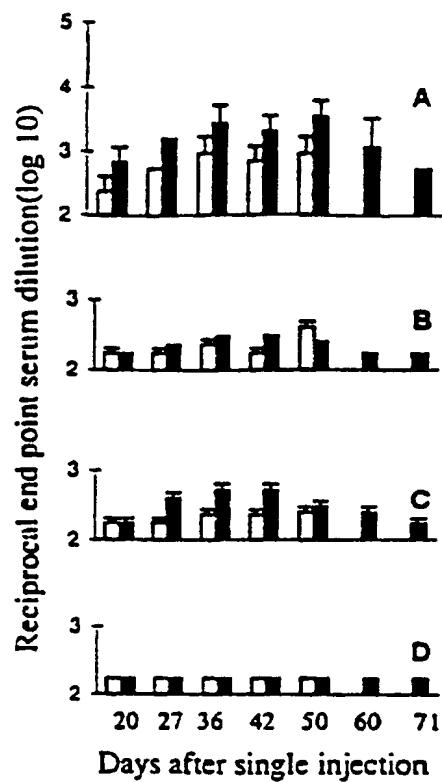


Figure 3

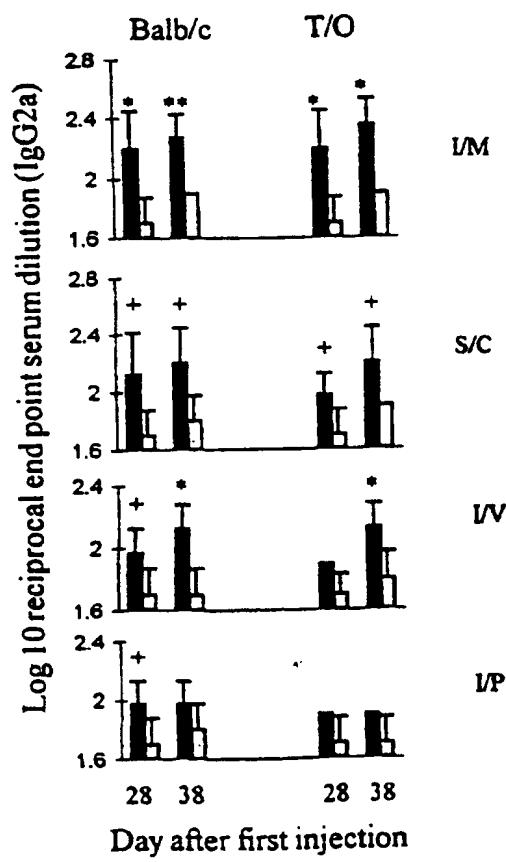


FIG. 5

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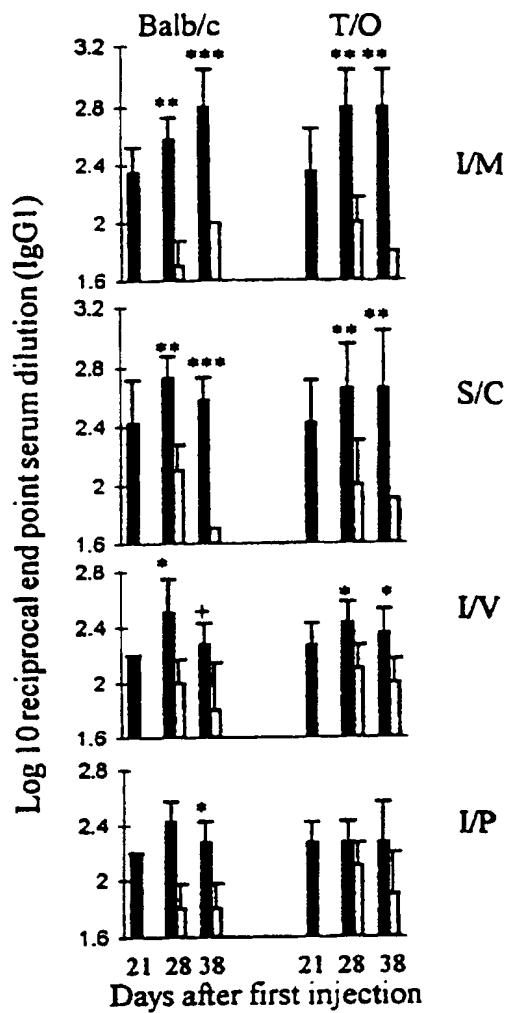


FIG. 4

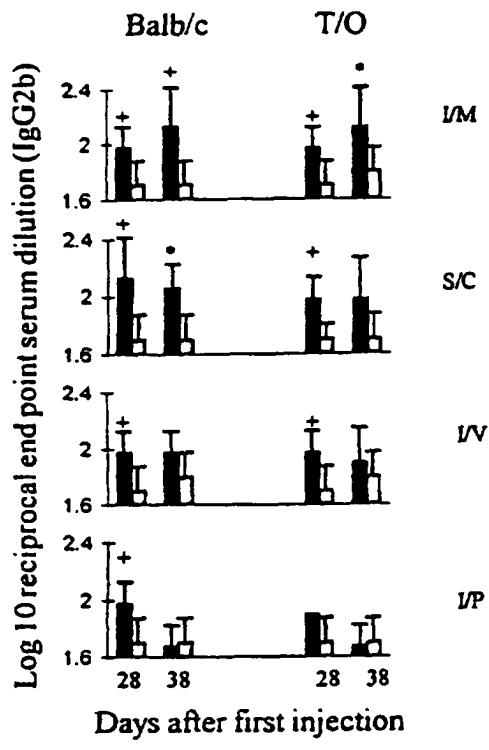


FIG. 6

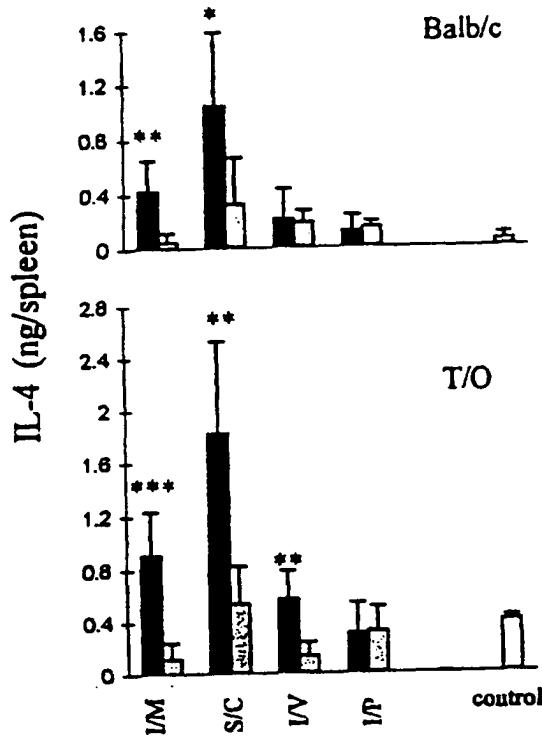


FIG. 7

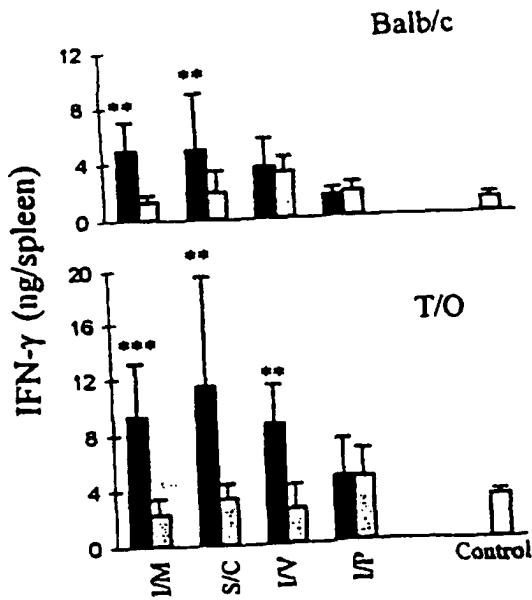


FIG. 8

A. CLASSIFICATION OF SUBJECT MATTER

A 61 K 9/127, A 61 K 39/00, A 61 K 48/00, C 12 P 25/00,
C 12 N 15/00

6

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A 61 K, C 12 N, C 12 P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 91/17424 A1 (VICAL, INC.) 14 November 1991 (14.11.91), abstract, claims 1,3,4,6, 9-11,12,18,26,27, page 20, line 26 - page 22, line 22, page 23, line 19 - page 25, line 16 (cited in the application). --	1-5, 7- 32
X	US 4897355 A (EPPSTEIN D.A. et al.) 30 January 1990 (30.01.90), abstract, claims 1,5,7-10, examples 5,7-10,18 (cited in the application). --	1-32
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Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search 11 December 1997	Date of mailing of the international search report 15.01.98
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HU Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer MAZZUCCO e.h.

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	KENKYUSHO) 18 March 1992 (18.03.92), the whole document. --	19,20, 25,26
X	Patent Abstracts of Japan, Vol. 14, No. 370 (C-747), 1990; & JP,A,02-135092 (BITAMIN KENKYUSHO K.K.), abstract. --	1-9, 16,19, 25,26
X	Patent Abstracts of Japan, Vol. 13, No. 238 (C-603), 1989; & JP,A,01-047381 (BITAMIN KENKYUSHO K.K.), abstract. --	1,2,14
X	WO 95/04524 A1 (OPPERBAS HOLDING B.V.) 16 February 1995 (16.02.95), abstract, claims 1,13-15, 20-22, page 18, third paragraph, fig. 3, example 3. --	1-9, 12,14- 24,26, 27
A	WO 95/13796 A1 (DEPOTECH CORPORATION) 26 May 1995 (26.05.95), claims 1,12,18,22-24,41, 43,44, page 7, lines 3-27, page 8, line 28 - page 9, line 9, page 9, lines 46-48, page 15, lines 8-25. -----	1-6, 11,14- 24

INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB 97/02490

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:

because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 20-24, 26, 27 and partially 25
is(are) directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition.

2. Claims Nos.:

because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:

3. Claims Nos.:

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.

3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

ANHANG

zum internationalen Recherchenbericht über die internationale Patentanmeldung Nr.

ANNEX

to the International Search Report to the International Patent Application No.

PCT/GB 97/02490 SAE 170125

In diesem Anhang sind die Mitglieder der Patentfamilie der im obengenannten internationalen Recherchenbericht angeführten Patentdokumente angegeben. Diese Angaben dienen nur zur Orientierung und erfolgen ohne Gewähr.

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The Office is in no way liable for these particulars which are given merely for the purpose of information.

au rapport de recherche international relatif à la demande de brevet international n°

ANNEXE

La présente annexe indique les membres de la famille de brevets relatifs aux documents de brevets cités dans le rapport de recherche international visé ci-dessus. Les renseignements fournis sont donnés à titre indicatif et n'engagent pas la responsabilité de l'Office.

Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
WO A1 9117424	14-11-91	AU A1 79794/91	27-11-91
US A 4897355	30-01-90	AT E 50983 AU A1 51853/86 AU B2 594654 CA A1 1288774 DE CO 3669503 EP A1 187702 EP B1 187702 JP A2 61161246 JP B4 6062517 JP A2 7070011 JP B2 2546623 NZ A 214716 ZA A 8600081 US A 4946787 US A 5049386 US A 5208036 US A 5266737 US A 5345412 US A 5550289 US A 5622712	15-03-90 17-07-86 15-03-90 10-09-91 19-04-90 16-07-86 14-03-90 21-07-86 17-08-94 14-03-95 23-10-96 26-04-89 26-08-87 07-08-90 17-09-91 04-05-93 22-11-94 13-08-96 27-08-96 22-04-97
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JP A2 2135092	23-05-90	keine - none - rien	
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WO A1 9504524	16-02-95	AU A1 73849/94 CA AA 2168671 EP A1 713388 IL A0 110305 JP T2 9501169	28-02-95 16-02-95 29-05-96 21-10-94 04-02-97
WO A1 9513796	26-05-95	AU A1 10535/95 BG A 100596 BR A 9408072 CA AA 217671 CN A 1140989 EP A1 729351 FI A0 962048 FI A 962048 HU A0 9601316 HU A2 75162 IL A0 111628 JP T2 9505301 NO A0 962024 NO A 962024 PL A1 314485 ZA A 9409063	06-06-95 31-12-96 12-08-97 26-05-95 22-01-97 04-09-96 14-05-96 15-07-96 29-07-96 28-04-97 24-01-95 27-05-97 15-05-96 09-07-96 16-09-96 13-09-95

